REMARKS/ARGUMENTS

This is in response to the Office Action mailed October 17, 2008 for the above-captioned application. Reconsideration and further examination are respectfully requested.

Claims 21-23 have been added to reflect specific embodiments of the invention. The claims are supported by the specification at paras. 50-55, and 67. No new matter has been added.

Enablement

Claims 1-3, 5, 7-9, 15, 17 and 19 stand rejected under 35 USC § 112, first paragraph, as lacking enablement. The Examiner asserts there is no enablement of a method wherein the therapeutic agent targets β4, or where the agent is an antibody that targets β4.

The Examiner states that the specification fails to show that agents that reduce the amount of active α6β4 and target and inhibit the signaling function of β4 inhibit angiogenesis. Applicants submit that the specification shows a relationship between inhibition of the signaling portion of β4 and inhibition of angiogenesis, and that one skilled in the art would understand that there are numerous ways to inhibit protein functions, including with antibodies.

The data given in the specification shows a relationship between inhibition of β4 and inhibition of angiogenesis. Experimental data showed that inhibition of the signaling/substrate portion of β4(the C-terminal end) leads to much lower complexity of vasculation in bFGF induced angiogenesis. Specification, para. 0057. This indicates that loss of β4 signaling impairs bFGF-induced angiogenesis to a significant extent. The data also showed that the substrate domain of β4 promotes endothelial cell migration and invasion in response to bFGF in human umbillical vein cells. Para. 0062 and 0063. In addition, the experimental data showed that β4 is expressed in significant levels in medium- and small-sized vessels of human papillary thyroid carcinoma, breast adenocarcinoma, prostate carcinoma, and glioblastoma multiforme. Specification, para. 0054. Finally, the data showed that loss of activity of the substrate domain of

β4 leads to reduced tumor growth in melanomas, lung carcinomas, lymphomas and fibrosarcomas, as well as corresponding reduction in microvessel density. Para. 0067.

In addition, the specification lists descriptions of effective antibodies and how to obtain them. It also lists numerous specific examples of antibodies for B4. *See* paras. 0029, 0039 anti-alpha-6 Mab CoH3 both human and mouse a6b1 and α6β4. Niessen et al., 211 (2) Exp Cell Res. 360-7 (Apr. 1994). The anti-beta-4 Mab ASC-3 blocks human beta-4 (Weaver et al., *beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium*, 2 Cancer Cell 205 (September 2002). Another known antibody is anti-beta-4 Mab 346-11A. (Zent et al. *"Involvement of laminin binding integrins and laminin-5 in branching morphogenesis of the ureteric bud during kidney development.*, 238 Dev Biol. 289 (Oct. 15 2001).

A person skilled in the art would also understand that phosphorylation of a protein may be inhibited by antibodies. For example, the anticancer drugs Trastuzumab and Pertuzumab, are directed to the Ectodomain of ErbB2 to inhibit activation of ErbB2 and thereby phosphorylation of its cytoplasmic domain. *See, e.g.,* Rita Nahta, *The HER-2-Targeting Antibodies Trastuzumab and Pertuzumab SynergisticallyInhibit the Survival of Breast Cancer* Cells, 64 CANCER RESEARCH 2343 (April 1, 2004). Inhibition of phosphorylation of β4 would eliminate the signaling function in a manner similar to the removal of the signaling portion of β4.

The Examiner has offered no evidence that a person skilled in the art would not understand how to use an antibody to inhibit protein function, nor that said person would be unable to inhibit phosphorylation of a target protein with an antibody. A person skilled in the art would be able to combine the teachings of the disclosure with the knowledge in the prior art to construct an antibody that would inhibit the signaling function of β4, thereby inhibiting angiogenesis, without undue experimentation.

The Examiner has cited Hiran as teaching that α6β4 is not expressed during developmental angiogenesis. As used in Hiran, developmental refers to pre-natal, ie, in the womb. Hiran, at 3777 (whisker pads were taken from E19.5 embryos). This does not teach

anything about post-natal angiogenesis. Hiran teaches that α6β4 is present post-natally and is a potential regulator of angiogenesis. *See* Hiran, at 3779. The fact that α6β4 is not present developmentally does not mean that a person skilled in the art would not know how to practice the invention. It merely possibly means that the invention may not work pre-natally.

The Examiner cites Sepp as teaching that inhibiting the function of β4 does not lead to inhibition of angiogenesis. Sepp merely discloses that use of two specific promoters of angiogenesis (bFGF and PMA) lead to a reduction of β4. The Examiner's argument ignores cause and effect, as well as the many reasons β4 may be reduced. The Examiner also ignores the statement that bFGF stimulation of bovine adrenal cortex endothelial cells induces an <u>increase</u> in β4 production. Sepp, at 270. In addition, as the Examiner has pointed out, the two functions of β4- adhesion and signaling- are quite separate. It is entirely plausible both that reduction of β4 adhesion would lead to angiogenesis and reduction of β4 signaling would lead to antiangiogenesis. Sepp does not provide any information about which <u>function</u> of β4 is reduced; it merely states that overall β4 is reduced. The experimental data provided in the specification shows that inhibition of signaling does have an antiangiogenic effect. The present invention concerns a reduction in the amount of signaling function of B4, not overall β4.

Because Applicants have shown that inhibition the signaling function of β4 has an antiangiogenic effect, and a person skilled in the art would be able to inhibit this function with an antibody, the rejection for lack of enablement is in error.

Written Description

Claims 1-3, 5, 7-9, 15, 17 and 19 stand rejected under 35 USC § 112, first paragraph, as lacking written description.

The Examiner points to the terms "tissue expressing α6β4", "agent that targets β4" and "pathological angiogenesis in a tissue expressing α6β4 integrin" and states that Applicants have not provided enough examples to constitute a representative number of species nor provided a description of structural features that are common to species within each genus.

As to "tissue expressing α6β4", the Examiner points to Hiran to state that α6β4 is not expressed during developmental angiogenesis and states that no species in the specification are shown to express α6β4 during angiogenesis. While Hiran does state that α6β4 is not expressed during developmental angiogenesis, this only refers to pre-natal tissue. It does nothing to show that "tissue expressing α6β4" is not defined. In fact, it proves that a person skilled in the art is in possession of methods of determining whether a tissue is expressing α6β4 by proving that a negative result can be found. In addition to teaching that α6β4 is not expressed during developmental angiogenesis, Hiran teaches methods of detecting expression, such as immunostaining. Hiran, 3772-73. Therefore, a person skilled in the art would understand the meaning of "tissue expressing α6β4," as the Applicants use it in describing the invention, and it is fully supported by a written description.

In addition, the assertion that the specification teaches no species that express α6β4 is incorrect. The specification teaches numerous such species. For example, at paragraphs 0054 - 0055, human papillary thyroid carcinoma, breast adenocarcinoma, prostate carcinoma, glioblastoma multiforme, and melanoma are all shown to express α6β4 in medium and small vessels.

It is not necessary to describe all types of tissue that may express α6β4 during angiogenesis. Sufficient examples are given such that a person skilled in the art may determine whether a tissue is expressing α6β4 and appreciate Applicants' recognition of the general nature of the invention.

As to "agent that targets β4", as stated previously, numerous antibodies, oligonucleotides, and binding proteins have been discussed and disclosed by the specification. These provide some examples of potential agents. A person skilled in the art would be able to look at these agents, as well as the knowledge that the C-terminal region controls signaling function, to determine characteristics of a broad range of agents that would target the known sequence of this region. ufficient diverse examples with similar activity have been provided to support this generic claim with written description.

As to "pathological angiogenesis in a tissue expressing α6β4 integrin", the specification provides methods of determining angiogenesis, such as with immunostaining. One target for this staining is PECAM-1, as described in para. 0054. Additionally, numerous tumor types that are associated with angiogenesis are described in para. 0067, such as melanoma cells, lung carcinoma cells, and lymphoma cells. Therefore, this term would be clear to one skilled in the art.

As stated previously, Sepp does not teach that inhibition of β4 does not lead to inhibition of angiogenesis. Sepp merely teaches that administration of angiogenic agents bFGF or PMA may reduce the amount of expressed β4. There is no indication that there is any causal relationship, nor is there a teaching of the function of β4 that is reduced. The experimental data given in the specification shows a relationship between inhibition of the signaling function of β4 and inhibition of angiogenesis.

Applicants submit that a person skilled in the art would understand the limits of the current claimed invention, and, utilizing this skill, would understand what potential agents and targets would be. It is not necessary to describe all potential targets, as sufficient structural limitations are given to describe these targets. Therefore, the rejection for lack of written description is in error.

Anticipation - Land

In addition, the Examiner has maintained his rejection under 35 USC § 102(b)/(e) for anticipation by Land (US Pat. Pub. 20030224993). Land teaches methods of inhibiting proliferation of certain cancer cells by contacting the β4 integrin with a composition that inhibits ligand binding. The Examiner states that antibodies are disclosed at paras. 47-48.

To anticipate, the prior art must teach all the claim elements <u>and the claimed arrangement</u>. "Section 102 embodies the concept of novelty—if a device or process has been previously invented (and disclosed to the public), then it is not new, and therefore the claimed invention is "anticipated" by the prior invention. . . . Because the hallmark of anticipation is prior invention, the prior art reference—in order to anticipate under 35 U.S.C. § 102—must not only disclose all

elements of the claim within the four corners of the document, but must also disclose those elements 'arranged as in the claim.'" Net MoneyIn v. Verisign, No. 07-1565 (Fed. Cir. 2008). In an anticipation rejection, "it is incumbent upon the examiner to identify wherein each and every facet of the claimed invention is disclosed in the applied reference." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (BPAI 1990).

Land does not teach all of the elements of the claimed invention. As the Examiner previously stated, the binding/adhesion function of β4 is quite separate from the signaling function, both spatially and temporally. Land merely teaches targeting of the binding portion, while teaching nothing about the signaling portion. This is the focus of the current invention, and Land teaches nothing about this.

In addition, Land teaches nothing about inhibiting angiogenesis. In the case of a method claim, a showing of anticipation requires that practicing the method described in the art would inherently (i.e. necessarily) achieve the undisclosed result which it the object of the claimed method, i.e. inhibiting angiogenesis. *Ex parte Levy*, 17 USPQ2d 1461, 1464 (BPAI 1990)(To establish anticipation under the theory of inherency, "the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art."). Land teaches inhibition of proliferation. The Examiner has given no evidence that targeting the β4 binding region would inherently result in inhibition of angiogenesis. As stated above, the two regions have very different functions and locations. There is no way that targeting of one would inherently target the other.

Finally, particular antibodies are not disclosed in this reference. While the cited paragraphs generally state that antibodies may be used to inhibit function, no specific targets are given. There is no sequence or structure given for the antibodies, nor is a target given. In addition, even if a target were given, the antibody would not necessarily be functional to inhibit the signaling function of β4 as the only region discussed in the reference is the binding region of β4.

The Examiner states that the prior art agents would necessarily target the signaling function of β4. According to *Fitzgerald*, the PTO must have a reason to believe a functional limitation is an inherent characteristic of the prior art before a *prima facie* case is established and it may require the applicant to prove the prior art does not possess that characteristic. *In re Fitzgerald*, 205 USPQ 594, 596, 597 (Fed. Cir. 1980). In *Fitzgerald*, this only arose when the prior art was identical or nearly identical. Here, there is no indication given that the binding region of the reference and the signaling region of the claimed invention are similar. In fact, as the Examiner has pointed out, these regions are quite distinct. Therefore, the Examiner has failed to establish a *prima facie* case that the prior art agents would target both regions.

As Land does not disclose all of the elements of the current claimed invention expressly or inherently, it does not anticipate, and this rejection is in error.

Anticipation - Bennett

Finally, the Examiner has maintained his rejection under 35 USC § 102(e) for anticipation by Bennett (US Pat. Pub. 20060172957). This reference concerns antisense oligonucleotides for modulating the expression of integrin β4 binding protein, also known as eIF6 and eIF3A. *See* Bennett, para. 6

The β4 binding protein of Bennett is not the integrin β4 subunit of the instant claims. The binding protein of Bennett binds to the β4 subunit in vitro. *See* Wikipedia.org (EIF6). It is not the same as the β4 subunit. Bennett does not describe modulation of the integrin β4 subunit of the instant claims and therefore does not anticipate.

In addition, Bennett does not mention any targeting of signaling function of β4, nor does it mention angiogenesis. Also, there is no disclosure of the use of any antibodies as referred to in claims 5, 9, 17, 19, and 21-23. As stated above, in order to disclose, structure or sequence must be disclosed. The only mention of antibodies in the reference is for detection purposes, primarily Western Blots. There is no disclosure of antibodies for inhibitory use, particularly at a therapeutic level similar to the antisense used.

The Examiner cites paragraph 9 as teaching antibodies as a therapeutic agent. The text of paragraph 9 reads: "Currently, there are no known therapeutic agents which effectively inhibit the synthesis of integrin beta 4 binding protein and to date, strategies aimed at investigating integrin beta 4 binding protein function have involved the use of antibodies. Consequently, there remains a long felt need for agents capable of effectively inhibiting integrin beta 4 binding protein function." This clearly states the opposite of what the Examiner asserts. There are no therapeutic antibodies for β4, antibodies for β4 have only been used for investigation, and there is a need for antibody agents. Antibodies are clearly not disclosed as therapeutic agents.

As Bennett clearly does not disclose all of the elements of the current claimed invention expressly or inherently, it does not anticipate, and this rejection is in error.

Respectfully submitted,

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EIF6

From Wikipedia, the free encyclopedia

edit (http://en.wikipedia.org/w/index.php?title=Template:PBB/3692&action=edit)

Eukaryotic translation initiation factor 6 (**EIF6**), also known as **Integrin beta 4 binding protein** (**ITGB4BP**), is a human gene.[1]

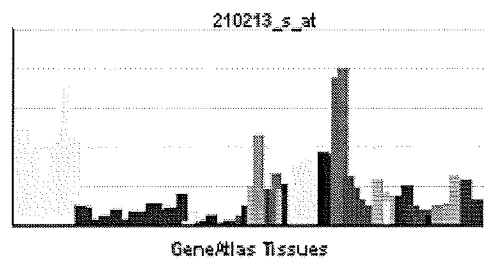
Eukaryotic translation initiation factor 6

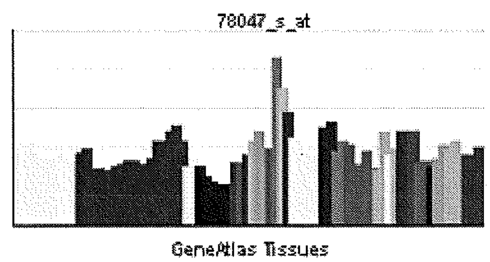
Identifiers

Symbols	EIF3A (http://www.genenames.org/data/hgnc_data.php?hgnc_id=6159) ; ITGB4BP; CAB; EIF6; b(2)gcn; p27BBP
External IDs	OMIM: 602912 (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=602912) MGI: 1196288 (http://www.informatics.jax.org/searches/accession_report.cgi?id=MGI:1196288) HomoloGene: 7135 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=homologene&dopt=HomoloGene&list_uids=7135)

Gene ontology [show]

RNA expression pattern





More reference expression data (http://symatlas.gnf.org/SymAtlas/symquery?q=EIF3A)

Orthologs

	Human	Mouse
Entrez	3692 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=retrieve&dopt=default&	16418 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=retrieve&dopt=default&

	list_uids=3692&rn=1)	list_uids=16418&rn=1)
Ensembl	ENSG00000126005 (http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000126005;db=core)	ENSMUSG00000027613 (http://www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG00000027613;db=core)
Uniprot	P56537 (http://www.expasy.org/uniprot/P56537)	Q3U740 (http://www.expasy.org/uniprot/Q3U740)
Refseq	NM_002212 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NM_002212) (mRNA) NP_002203 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_002203) (protein)	NM_010579 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NM_010579) (mRNA) NP_034709 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_034709) (protein)
Location	Chr 20: 33.33 - 33.34 Mb (http://genome.ucsc.edu/cgi-bin/hgTracks?org=Human&position=chr20:33330128-33336202)	Chr 2: 155.51 - 155.52 Mb (http://genome.ucsc.edu/cgi-bin/hgTracks?org=Mouse&position=chr2:155511278-155518366)
Pubmed search	[1] (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Link&LinkName=gene_pubmed&from_uid=3692)	[2] (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Link&LinkName=gene_pubmed&from_uid=16418)

Hemidesmosomes are structures which link the basal lamina to the intermediate filament cytoskeleton. An important functional component of hemidesmosomes is the integrin beta-4 subunit (ITGB4), a protein containing two fibronectin type III domains. The protein encoded by this gene binds to the fibronectin type III domains of ITGB4 and may help link ITGB4 to the intermediate filament cytoskeleton. The encoded protein, which is insoluble and found both in the nucleus and in the cytoplasm, can function as a translation initiation factor and prevent the association of the 40S and 60S ribosomal subunits. Multiple transcript variants encoding several different isoforms have been found for this gene.[1]

References

1. ^ *a* *b* "Entrez Gene: ITGB4BP integrin beta 4 binding protein (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=3692) ".

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[CANCER RESEARCH 64, 2343–2346, April 1, 2004]

Advances in Brief

The HER-2-Targeting Antibodies Trastuzumab and Pertuzumab Synergistically Inhibit the Survival of Breast Cancer Cells

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Abstract

Trastuzumab (herceptin) and pertuzumab (Omnitarg, 2C4) are recombinant humanized monoclonal antibodies that target different extracellular regions of the HER-2 tyrosine kinase receptor. We explored combination effects of these agents in the HER-2-overexpressing BT474 breast cancer cell line. Trastuzumab and 2C4 synergistically inhibited the survival of BT474 cells, in part, because of increased apoptosis. Trastuzumab increased 2C4-mediated disruption of HER-2 dimerization with the epidermal growth factor receptor and HER-3. Combination drug treatment reduced levels of total and phosphorylated HER-2 protein and blocked receptor signaling through Akt but did not affect mitogen-activated protein kinase. These results suggest that combining HER-2-targeting agents may be a more effective therapeutic strategy in breast cancer rather than treating with a single HER-2 monoclonal antibody.

Introduction

The *her-2* (*erbB-2, neu*) gene encodes a M_r 185,000 transmembrane glycoprotein that is a member of the epidermal growth factor receptor (EGFR or erbB) family of receptor tyrosine kinases. As the preferred heterodimerization partner among ligand-bound EGFR family members, HER-2 mediates lateral signal transduction, resulting in mitogenesis, apoptosis, angiogenesis, and cell differentiation (1). The *her-2* gene is amplified and overexpressed in ~20–30% of invasive breast carcinomas, and is associated with increased metastatic potential and decreased overall survival (1, 2).

Trastuzumab (herceptin; Genentech, San Francisco, CA) is a recombinant humanized monoclonal antibody directed against the extracellular domain of the HER-2 tyrosine kinase receptor. Clinical studies established that trastuzumab is active against HER-2-overexpressing metastatic breast cancers, leading to its approval in 1998 by the United States Food and Drug Administration (3). The objective response rates to trastuzumab monotherapy range from 12 to 34% for a median duration of 9 months (4). Current treatment regimens combining trastuzumab with the taxane paclitaxel (5, 6) or docetaxel (7) increase response rates, time to progression, and survival.

Another HER-2-targeted monoclonal antibody, pertuzumab (Omnitarg, 2C4; Genentech), is currently being tested in Phase I clinical trials in cancer patients with different types of solid tumors. In contrast to trastuzumab, pertuzumab sterically blocks HER-2 dimerization with other HER receptors and blocks ligand-activated signaling from HER-2/EGFR and HER-2/HER-3 heterodimers (8). As the majority of breast tumors that initially respond to trastuzumab begin to progress again within ~1 year (4, 6), treatment with combined HER-2-targeting strategies may be beneficial. We demonstrate here that trastuzumab and pertuzumab synergistically block the survival of HER-2-overexpressing BT474 breast cancer cells.

Materials and Methods

Materials. Trastuzumab (Genentech) was dissolved in sterile water at 20 mg/ml. Pertuzumab (Genentech) was provided in sterile water at 25 mg/ml. The MTS CellTiter 96 Aqueous One Solution proliferation reagent (Promega, Madison, WI) was used in accordance with manufacturer guidelines. The caspase inhibitor Z-VAD-FMK was purchased from Promega Corporation (Madison, WI). Annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) were purchased from Becton Dickinson (Franklin Lakes, NJ).

Cell Culture. BT474 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with 10% FCS.

Dose-Response Studies. BT474 cells were seeded at 5×10^4 cells/well in 12-well dishes. After 24 h, cells were treated in triplicate with 2-fold serial dilutions of trastuzumab, pertuzumab, or both drugs simultaneously at a fixed 1:1 ratio at low doses ranging from 0.9 ng/ml to 10 ng/ml. After 5 days, cells were trypsinized and counted by trypan blue exclusion. Growth inhibition was calculated as the percentage of viable cells compared with untreated cultures. Combination indices (C.I.s) were obtained using the method of Chou and Talalay through the commercial software package Calcusyn (Biosoft, Cambridge, United Kingdom; Ref. 9). Results of trypan blue assays were confirmed by MTS assay as directed by the manufacturer. Briefly, BT474 cells were seeded at 1×10^3 cells/well in 96-well dishes. After 24 h, cells were treated in triplicate with 2-fold serial dilutions of trastuzumab, pertuzumab, or both drugs simultaneously at a fixed 1:1 ratio using the same concentrations as in trypan blue assays. In some cases, the caspase inhibitor Z-VAD-FMK was added at a fixed 20-μM concentration. After 5 days, cells were exposed to the MTS reagent, and absorbance was measured in a microplate reader. Growth inhibition was calculated as the percentage of proliferating cells compared with untreated cultures.

Cell Cycle Analysis. BT474 cells were treated in duplicate with control IgG1 (10 μg/ml; Calbiochem, San Diego, CA) or with 0.1, 1, 10, or 100 μg/ml trastuzumab and/or 0.1, 1, 10, or 100 μg/ml pertuzumab. After 5 days of drug treatment, cells were fixed overnight in 70% ethanol and were resuspended in propidium iodide (50 μg/ml) supplemented with RNase A (1 μg/ml). DNA content was measured using a FACScan cytometer (Becton Dickinson). To specifically assess cell death, cells were treated with 0.1, 1, 10, or 100 μg/ml trastuzumab and/or pertuzumab, and were stained with annexin V-PE and 7-AAD after 5 days of drug treatment. Cell death was measured as cells staining positive for annexin V, 7-AAD, or both, as assessed by fluorescence-activated cell sorting analysis.

Immunoblotting. BT474 cells were treated with 0.1, 1, or 10 μg/ml trastuzumab and/or 0.1, 1, or 10 μg/ml pertuzumab. Protein lysates were obtained after 5 days of drug treatment using 1% NP40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8), and 1% NP40) and immunoblotted (25 μg) for poly(ADP-ribose) polymerase (PARP; polyclonal), HER-2 (monoclonal Ab-3; Oncogene Research Products, La Jolla, CA), phospho-HER-2, phospho-serine 473 Akt (monoclonal), total Akt (polyclonal), phospho-threonine 202/tyrosine 204 p44/p42 mitogen-activated protein kinase (MAPK; polyclonal), and total MAPK (polyclonal). All of the primary antibodies were from Cell Signaling Technology (Beverly, MA), unless otherwise specified, and were used at a 1:1000 dilution in 5% nonfat dried milk in PBS-Tween (PARP, total Akt, and total MAPK) or TRIS-buffered-saline-Tween (phospho-specific antibodies). Secondary antibodies

Received 12/9/03; revised 1/22/04; accepted 2/18/04.

Grant support: Supported in part by the Nellie B. Connally Breast Cancer Research Fund. F. J. Esteva is a recipient of a Career Development Award from the National Cancer Institute (K23 CA82119).

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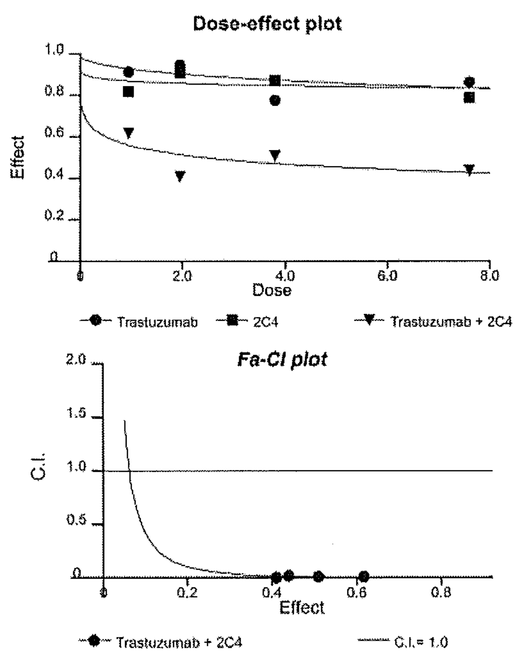
Requests for reprints: Francisco J. Esteva, Department of Breast Medical Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 424, Houston, TX 77030-4009. Phone: (713) 792-2817; Fax: (713) 745-5768; E-mail: festeva@mdanderson.org.

were chosen according to species of origin and were detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation. BT474 cells were treated with 1 μg/ml trastuzumab and/or 1 μg/ml pertuzumab. Protein lysates were obtained after 24, 48, or 72 h of drug exposure. HER-2 protein (200 μg) was immunoprecipitated from lysates using a monoclonal antibody conjugated to agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates and total protein lysates were immunoblotted using the following antibodies at 1:1000 dilution: EGFR polyclonal (clone 1005; Santa Cruz Biotechnology), HER-3 polyclonal (C-17; Santa Cruz Biotechnology), and β-actin (polyclonal; Santa Cruz Biotechnology). Secondary antibodies were chosen according to species of origin and were detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech).

Results and Discussion

Trastuzumab and Pertuzumab Synergistically Inhibit the Growth of Breast Cancer Cells. HER-2-overexpressing BT474 cells were treated in triplicate with 2-fold serial dilutions of trastuzumab and/or pertuzumab at a fixed 1:1 ng/ml drug concentration ratio. Inhibition of cell survival was examined by trypan blue exclusion after 5 days of treatment, and was calculated as the percentage of viable cells relative to untreated cell cultures. Dose-response experiments were repeated three times for statistical validity. A representative dose-effect plot shows that the combination of trastuzumab and pertuzumab mediates a loss of up to 60% of cells at doses in which individual drugs do not alter cell survival (Fig. 1). Data were analyzed using the method of Chou and Talalay (9) to establish drug C.I. values. Statistically, drug synergy, addition, and antagonism are defined by C.I. values less than 1.0, equal to 1.0, or greater than 1.0, respectively. A plot of the fraction of affected cells *versus* C.I. indicates that trastuzumab and pertuzumab interact synergistically, because C.I. values are below 1.0. Furthermore, C.I. values less than 0.1, as are seen in these experiments, indicate very strong synergism between drugs. Drug synergy was further confirmed by MTS assay (not shown), showing that these antibodies synergistically inhibit the proliferation of BT474 breast cancer cells. These results demonstrate that trastuzumab and pertuzumab are highly synergistic inhibitors of BT474 breast cancer cell survival.



Combination Index (C.I.) Values for Trastuzumab + 2C4 (1:1):

ED_{50}	ED_{75}	ED_{90}	D_m	m	r
0.00983	0.00159	0.00486	2.49063	−0.25841	0.61434

Fig. 1. Trastuzumab and pertuzumab synergistically inhibit the survival of BT474 breast cancer cells. BT474 cells were treated in triplicate with 2-fold serial dilutions of trastuzumab, 2C4 (pertuzumab), or both drugs simultaneously at a fixed 1:1 ratio at low doses ranging from 0.9 to 10 ng/ml. After 5 days, cells were trypsinized and were counted by trypan blue exclusion. Growth inhibition was calculated as the fraction of viable cells compared with untreated cultures. Dose-effect and fraction affected *versus* combination index plots (*Fa-CI plots*) were generated using the method of Chou and Talalay with the commercial software package Calcusyn (Biosoft, Cambridge, UK). Doses on the X axis are stated in ng/ml. C.I. values are listed for effective doses at which 50, 75, or 90% (ED_{50}, ED_{75}, and ED_{90}, respectively) of cells are killed. Statistically drug synergy is defined by C.I. values less than 1.0, and very strong synergy is defined by C.I. values less than 0.1. D_m, the median-effect (ED_{50}) drug concentration as 2.5 ng/ml; $m < 1$ indicates a negative sigmoidal shape to the dose-effect curve; r states the linear correlation coefficient of the median-effect plot.

Trastuzumab and Pertuzumab Inhibit Proliferation and Induce Apoptosis. Flow cytometric cell cycle analysis was performed to determine whether the results of the dose-response assays were a reflection of cytostatic or cytotoxic effects due to cell cycle arrest or apoptosis. BT474 cells were treated in duplicate with a control IgG or with 100 μg/ml trastuzumab and/or pertuzumab. After 5 days of drug treatment, cells were fixed and resuspended in propidium iodide, and DNA content was measured in comparison with untreated cells (Fig. 2*A*). Trastuzumab alone induced a 3-fold increase in the subdiploid cell population, whereas 2C4 had no effect. Together, trastuzumab and 2C4 increased the subdiploid fraction by 12-fold. In addition, the combination of trastuzumab and 2C4 reduced the percentage of proliferating (S-phase) cells by more than 2-fold. Alterations in the percentages of G_1 and G_2-M cells were not significant, because similar changes were noted for cells treated with control IgG. Similar effects on the cell cycle were observed with lower concentrations of drugs at 0.1, 1, and 10 μg/ml. These flow cytometry results are consistent with dose-response experiments, indicating that a combination of trastuzumab and pertuzumab inhibits cell proliferation and survival to a greater degree than does either agent alone.

Cells were treated with 0.1, 1, 10, or 100 μg/ml concentrations of each agent or a control IgG for 5 days and were stained with annexin V-PE and 7-AAD to further assess cell death. Annexin V-PE binds to cells in early apoptosis and the fluorescent dye 7-AAD stains cells in late stages of apoptosis or cells that are already dead (10, 11). The percentages of cells staining positive for annexin V-PE and/or 7-AAD increased up to 5-fold as the concentration of drugs increased (Fig. 2*B*), indicating that trastuzumab and pertuzumab induce apoptotic cell death.

Poly(ADP-ribose) polymerase (PARP), which is cleaved by caspases during apoptosis to produce M_r 89,000 and 24,000 fragments from the full-length M_r 116,000 protein (12), was measured as a marker of apoptosis by immunoblotting. BT474 cells were treated with 0.1, 1, or 10 μg/ml trastuzumab and/or 2C4, and total protein was immunoblotted for PARP after 5 days of drug treatment (Fig. 2*C*). PARP fragments were detected in all drug-treated samples and were most abundant at the highest dose combination used (10 μg/ml concentration of each agent). These data confirm increased apoptosis in cells treated with a combination of trastuzumab and pertuzumab. In addition, the pan-caspase inhibitor Z-VAD-FMK blocked synergy between trastuzumab and pertuzumab, as measured by MTS proliferation assays (not shown), further establishing that the synergistic cytotoxicity achieved by this drug combination is due in part to apoptosis.

Trastuzumab Increases 2C4-Mediated Disruption of HER-2 Dimerization and Downstream Akt Signaling. BT474 cells were treated with 1 μg/ml trastuzumab and/or 2C4 and were lysed for protein after 24, 48, or 72 h. HER-2 was immunoprecipitated from each lysate and was immunoblotted for EGFR and HER-3 to determine effects on receptor dimerization (Fig. 3A). Combination trastuzumab-2C4 reduced HER-2 levels within 24 h to a greater degree than either single agent. Pertuzumab was previously reported to disrupt HER-2 receptor dimerization with EGFR and HER-3 (8). Although trastuzumab alone did not significantly alter levels of EGFR or HER-3 complexed to HER-2, 2C4 reduced the levels of these HER-2 dimers by 72 h. Trastuzumab in-

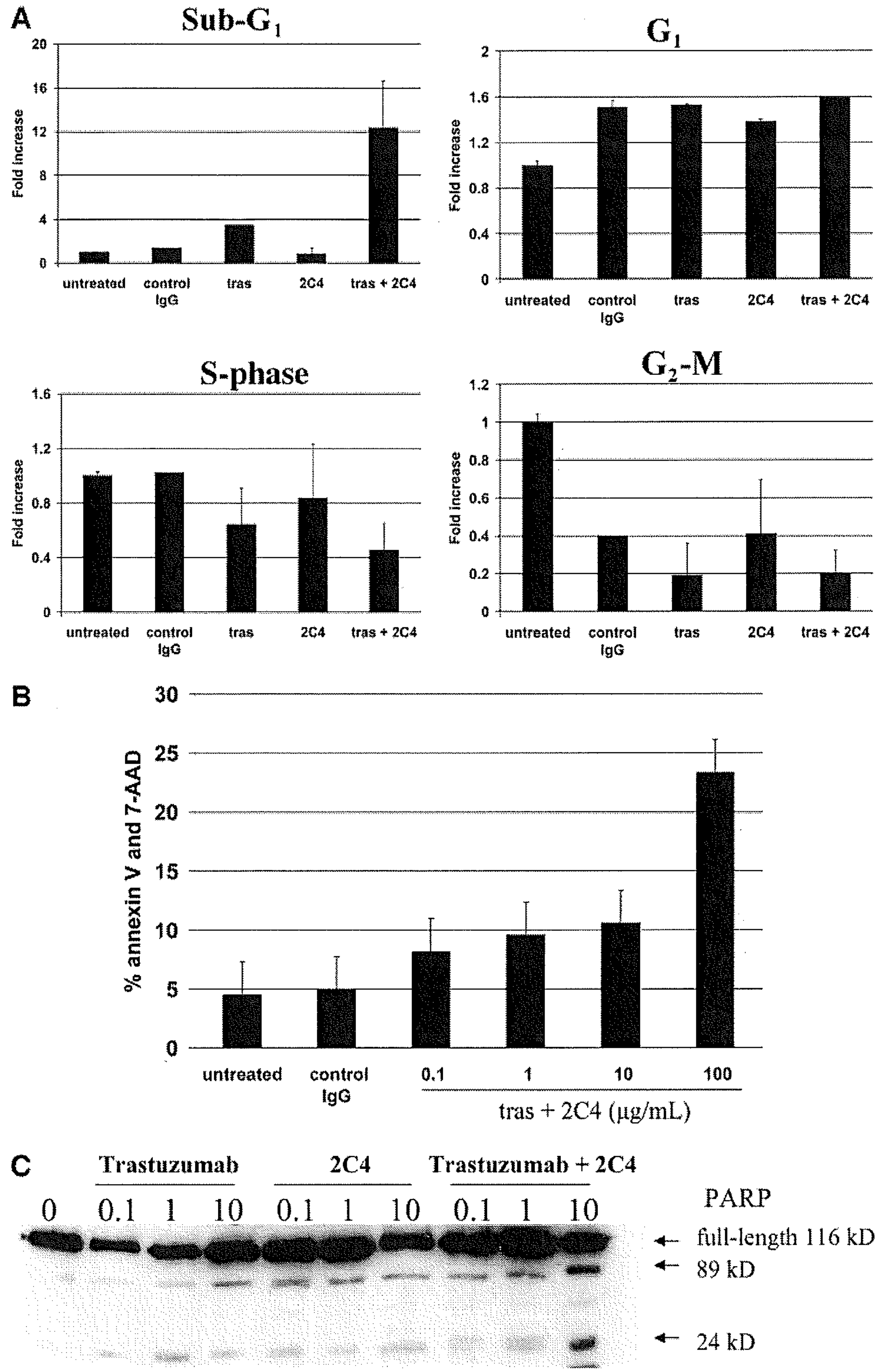


Fig. 2. Trastuzumab and pertuzumab induce apoptosis. *A*, BT474 cells were treated in duplicate with control (*IgG*) or with 100 μg/ml of trastuzumab (*tras*) and/or pertuzumab (*2C4*), cells were fixed, stained with propidium iodide, and measured for DNA content with a FACScan cytometer. The fold-increase in sub-G_1 (subdiploid), G_1, S-phase, and G_2-M cell percentages are shown. *B*, BT474 cells were treated in duplicate with control IgG or with 0.1, 1, 10, or 100 μg/ml of trastuzumab and/or 2C4. After 5 days of drug treatment, cells were stained with annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (*7-AAD*) and were analyzed for cell death on a FACScan cytometer. The percentages of cells staining positive for annexin V-PE and/or 7-AAD are shown. *C*, BT474 cells were treated in duplicate with 0.1, 1, or 10 μg/ml trastuzumab (*tras*) and/or 2C4. Protein lysates were obtained after 5 days of drug treatment and were immunoblotted (25 μg) with a poly(ADP-ribose) polymerase (*PARP*) polyclonal antibody. Cleaved PARP is represented by M_r 89,000 and 24,000 fragments, and full-length PARP is M_r 116,000. *kD*, M_r in thousands.

creased 2C4-mediated disruption of receptor dimers, because little or no EGFR and HER-3 were detectable on immunoblots by 72 h of combination drug treatment. This effect could not be due to down-regulation of HER-2 alone, as HER-2 was still detected in all of the combination drug samples at 72 h. Because total EGFR and HER-3 levels did not change on treatment with the drugs, these data suggest that trastuzumab increases the ability of pertuzumab to disrupt HER-2 receptor dimers. Furthermore, a dose-dependent down-regulation of total and phosphorylated HER-2 receptor levels was achieved by the combination of antibodies, primarily, because of 2C4 (Fig. 3*B*).

Signaling pathways activated by HER-2 include the phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK cascades. The combination of trastuzumab and 2C4 reduced levels of active phospho-serine 473 Akt to a greater degree *versus* either agent alone when administered at doses as low as 0.1 μg/ml for 72 h (Fig. 4). In contrast, signaling from the MAPK cascade was not inhibited, because levels of phosphorylated p44/p42 MAPK were unaltered by the combination of drugs.

Members of the erbB family of receptors are rarely, if ever, expressed alone, and HER-2 is frequently activated because of its role as a common coreceptor (8). The antitumor activity of trastuzumab is

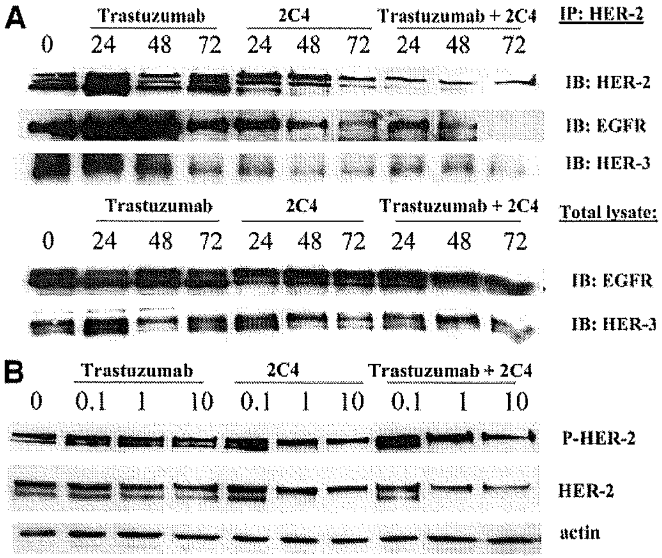


Fig. 3. Trastuzumab increases pertuzumab-mediated disruption of HER-2 dimers. BT474 cells were treated with 1 μg/ml trastuzumab and/or 1 μg/ml pertuzumab (2C4). Protein lysates were obtained after 24, 48, or 72 h of drug exposure. *A*, HER-2 protein (200 μg) was immunoprecipitated from total lysates using an agarose-conjugated monoclonal antibody. Immunoprecipitates (*IP*) were immunoblotted (*IB*) for HER-2, epidermal growth factor receptor (*EGFR*), and HER-3. Total protein lysates were also immunoblotted for total EGFR and HER-3. *B*, BT474 cells were treated with 0.1, 1, or 10 μg/ml trastuzumab and/or 2C4 for 5 days, and total protein lysates (50 μg) were immunoblotted for phosporylated (*P-HER-2*) and total HER-2.

believed to be due in part to HER-2 down-regulation with subsequent inhibition of downstream signaling and antibody-mediated immune function. However, trastuzumab does not affect signaling from other erbB receptors, nor does it inhibit ligand-activated signaling. The HER-2-targeted antibody 2C4, in contrast, inhibited heregulin-activated signaling in breast and prostate cancer models *in vitro* and *in vivo* because of dissociation of HER-2/HER-3 dimers (8, 13). Hence, dual treatment with both of the HER-2-targeted antibodies combines different mechanisms of growth inhibition. The result is synergistic cell death due at least in part to enhanced disruption of receptor dimers and reduced signaling from the Akt cell survival pathway. However, many questions regarding potential efficacy of this drug combination remain unanswered. Most importantly, we must determine what cell populations are most sensitive to combined trastuzumab-2C4 treatment. This may require developing tests to determine the level of HER-2 receptor dimers and the degree of ligand-activated signaling in individual tumors.

Trastuzumab monotherapy offers clinical benefit to a subset of

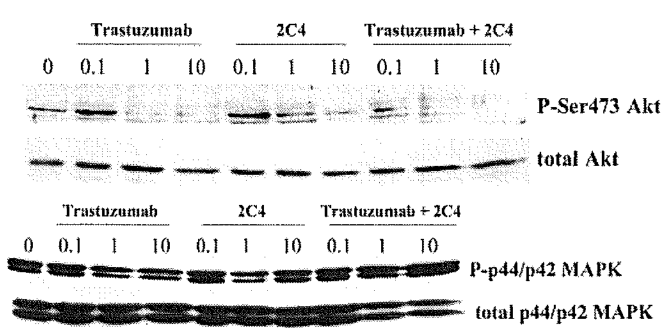


Fig. 4. Trastuzumab and pertuzumab (*2C4*) inhibit Akt signaling. BT474 cells were treated with 0.1, 1, or 10 μg/ml trastuzumab and/or 2C4 for 5 days. Total protein lysates (50 μg) were immunoblotted for phosporylated serine 473 Akt (*P-Ser473 Akt*), total Akt, phosphorylated p44/p42 mitogen-activated protein kinase (*P-p44/p42 MAPK*), and total MAPK (*total p44/p42 MAPK*).

HER-2-overexpressing metastatic breast cancers. However, the majority of breast cancers that initially respond to trastuzumab-containing regimens begin to progress again within 1 year (14). Thus, the potential efficacy of combination trastuzumab-2C4 in trastuzumab-resistant breast cancer cells is the focus of ongoing studies. Additionally, the effect of combining 2C4 with other erbB-targeted agents including tyrosine kinase inhibitors is of interest. Such combinations may prove to be effective not only in HER-2-overexpressing breast cancer cells, but also in cancers that overexpress EGFR or erbB ligands, as both 2C4 and tyrosine kinase inhibitors inhibit EGFR and ligand-activated signaling. Combining 2C4 with other conventional treatments such as chemotherapies may also reveal potential sensitization, as has been seen with other HER-2-targeting approaches (15). Additionally, evaluating combinations of 2C4 with antibodies that target other receptors such as EGFR or insulin-like growth factor-1 receptor (IGF-1R) is important, because these may demonstrate additive or synergistic effects. Other HER-2-targeting antibodies have demonstrated synergy *in vivo* using BT474 breast cancer xenografts (16, 17), directly supporting the findings of our present study. In conclusion, our results suggest that the combination of trastuzumab and pertuzumab may more effectively inhibit breast cancer cell survival *versus* trastuzumab alone.

Acknowledgments

We thank Genentech for providing 2C4, and Dr. Mark X. Sliwkowski for his suggestions and helpful advice.

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Anti-Integrin β4, clone ASC-3

Recommended Replacement for: 05-283

Species Reactivity	Key Applications	Host	Format	Antibody Type
H	FC, IP, IH, INHIB	Mouse	Purified	Monoclonal Antibody

UniProt Number:
P16144
UniProt Summary
FUNCTION: SwissProt: P16144 # Integrin alpha-6/beta-4 is a receptor for laminin. It plays a critical structural role in the hemidesmosome of epithelial cells.
SIZE: 1822 amino acids; 202151 Da
SUBUNIT: Heterodimer of an alpha and a beta subunit. Beta-4 associates with alpha-6.
SUBCELLULAR LOCATION: Membrane; Single-pass type I membrane protein.
TISSUE SPECIFICITY: Integrin alpha-6/beta-4 is predominantly expressed by epithelia. Isoform beta-4D is also expressed in colon and placenta. Isoform beta-4E is also expressed in epidermis, lung, duodenum, heart, spleen and stomach.
DOMAIN: SwissProt: P16144 The fibronectin type-III-like domains bind BPAG1 and plectin and probably also recruit BP230.
DISEASE: SwissProt: P16144 # Defects in ITGB4 are a cause of epidermolysis bullosa letalis with pyloric atresia (EB-PA) [MIM:226730]; also known as junctional epidermolysis bullosa with pyloric atresia (PA-JEB) or aplasia cutis congenita with gastrointestinal atresia. EB-PA is characterized by mucocutaneous fragility and gastrointestinal atresia, which most commonly affects the pylorus. & Defects in ITGB4 are a cause of generalized atrophic benign epidermolysis bullosa (GABEB) [MIM:226650]. This nonlethal form of junctional epidermolysis bullosa is characterized by life- long blistering of the skin, associated with hair and tooth abnormalities.
SIMILARITY: Belongs to the integrin beta chain family. & Contains 1 Calx-beta domain. & Contains 4 fibronectin type-III domains. & Contains 1 VWFA domain. **hide »**
Entrez Gene Number:
NM_000213.3
Entrez Gene Summary
Integrins are heterodimers comprised of alpha and beta subunits, that are noncovalently associated transmembrane glycoprotein receptors. Different combinations of alpha and beta polypeptides form complexes that vary in their ligand-binding specificities. Integrins mediate cell-matrix or cell-cell adhesion, and transduced signals that regulate gene expression and cell growth. This gene encodes the integrin beta 4 subunit, a receptor for the laminins. This subunit tends to associate with alpha 6 subunit and is likely to play a pivotal role in the biology of invasive carcinoma. Mutations in this gene are associated with epidermolysis bullosa with pyloric atresia. Multiple alternatively spliced transcript variants encoding distinct isoforms have been found for this gene.
hide »

Description:
Anti-Integrin β4, clone ASC-3
Clone:
ASC-3
Trade Name:
Chemicon (Millipore)
Key Applications:

- Flow Cytometry
- Immunoprecipitation
- Immunohistochemistry
- Inhibits Activity/Function

Application Notes:
Inhibition of cell attachment: inhibits SCC9 cell adhesion to human

laminin and laminin GD-2 peptide, but not to EHS laminin, type IV collagen or fibronectin. Does not inhibit SKOV-3 cell adhesion to laminin, fibronectin or type IV collagen.

Immunohistochemistry: on acetone-fixed frozen human epithelial tissues including tongue, foreskin and ovary. Not for use on formalin-fixed tissue.

Flow cytometry: stains human squamous cell carcinoma (SCC9), ovarian carcinoma (SKOV-3) and umbilical vein endothelium (HUVEC). Stains RKO or K562 cells transfected for CD104 expression. Does not stain monocytes, neutrophils, lymphocytes, T cells, B cells or platelets.

Immunoprecipitation: precipitates PAGE bands of 66, 76, 120, 135 and 200kDa (reduced) or 66, 110, 125, 130 and 190kDa (non-reduced) from surface-biotinylated SCC9 cells.

Working dilutions must be determined by end user.
Species Reactivity:
Human
Usage Statement:
Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.
Alternate Names:
CD104
Antibody Sub-Category:
Integrins
Concentration:
1mg/mL
Specificity:
Human beta4 integrin (CD104)
Antibody Category:
ECM & Adhesion
Gene Symbol:

- ITGB4
- CD104
- GP150

Format:
Purified
Presentation:
Liquid in 0.02M PB pH 7.6, 0.25M NaCl containing 0.1% sodium azide.
Isotype:
IgG1κ
Immunogen:
Human squamous cell carcinoma cells (SCC9)
Antibody Type:
Monoclonal Antibody
Replaces:
05-283
Storage Conditions:
Maintain at 2-8°C in undiluted aliquots for up to 6 months.
Host:
Mouse

Product Resources

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- Data Sheets
- FAQs
- MSDS

View Gene Related Products for this Target

- Antibodies (6)

Compatible Secondary Antibodies

Find Secondary Antibodies

Select the host, conjugate, and species, and then press Search to find the secondary antibodies for your research needs.

Trade Name - All -

Primary Antibody Host Mouse (130)

Conjugate - All -

Secondary Antibody Host - All -

Search clear

Product Support

- Custom Services Quote
- Bulk Services Quote
- Technical Service
- Customer Service

Anti-Integrin β4, clone 3E1

Species Reactivity	Key Applications	Host	Format	Antibody Type
H	FC, IP, IC, IH	Mouse	Ascites	Monoclonal Antibody

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P16144
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hide »

Description:
Anti-Integrin β4, clone 3E1
Clone:
3E1
Trade Name:
Chemicon (Millipore)
Background Information:
Integrin beta 4 is a glycoprotein which associates with the a6 integrin to form the a6/b4 complex. Integrin alpha 6/beta 4 is a receptor for laminin. It plays a critical structural role in the hemidesmosome of epithelial cells. Defects in Integrin beta 4 gene are a cause of epidermolysis bullosa letalis with pyloric atresia (EB PA); also known as junctional epidermolysis bullosa with pyloric atresia (PA-JEB) or aplasia cutis congenita with gastrointestinal atresia. EB-PA is characterized by mucocutaneous fragility and gastrointestinal atresia, which most commonly affects the pylorus. Moreover, defects in Integrin beta 4 gene are a cause of generalized atrophic benign epidermolysis bullosa (GABEB). This nonlethal form of junctional epidermolysis bullosa is characterized by life long blistering of the skin, associated with hair and tooth abnormalities.
Key Applications:

- Flow Cytometry
- Immunoprecipitation
- Immunocytochemistry
- Immunohistochemistry

Application Notes:
Stains human skin in immunofluorescence at approximately 1:200; acetone fixation required.

Also suitable for use in immunoprecipitation and flow cytometric assays.

Optimal working dilutions must be determined by the end user.
Species Reactivity:
Human
Usage Statement:
Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.
Alternate Names:
CD104
Antibody Sub-Category:
Integrins
Concentration:
Variable
Purification Method:
UnPurified
Specificity:
Reacts with Human beta4 integrin. The antigen is present on epithelial cells.
Antibody Category:
ECM & Adhesion
Gene Symbol:

- ITGB4
- CD104
- GP150

Format:
Ascites
Presentation:
UnPurified ascites containing no preservatives.
Isotype:
IgG1
Format Code:
Asc
Antibody Type:
Monoclonal Antibody
Control:
A431 cell lysate
Storage Conditions:
Maintain for 2 years at -20°C from date of shipment. Aliquot to avoid repeated freezing and thawing. For maximum recovery of product, centrifuge the original vial after thawing and prior to removing the cap.
Host:
Mouse

Product Resources

- Bibliography
- Data Sheets
- FAQs
- MSDS

View Gene Related Products for this Target

- Antibodies (6)

Compatible Secondary Antibodies

Find Secondary Antibodies

Select the host, conjugate, and species, and then press Search to find the secondary antibodies for your research needs.

Trade Name - All -

Primary Antibody Host Mouse (130)

Conjugate - All -

Secondary Antibody Host - All -

Search clear

Product Support

- Custom Services Quote
- Bulk Services Quote
- Technical Service
- Customer Service
